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Parkville Virus: A Novel Genetic Variant of Human Calicivirus in the Sapporo Virus Clade, Associated With an Outbreak of Gastroenteritis in Adults

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This report describes the characterization of Parkville virus, the etiologic agent of an outbreak of foodborne gastroenteritis, that has the morphology of a calicivirus and genetic properties that distinguish it from previously identified strains in the Sapporo/Manchester virus clade. Sequence analysis of the Parkville virus genome showed it contained the RNA-dependent RNA polymerase motifs GLPSG and YGDD characteristic of members of the family *Caliciviridae* with an organization identical to that reported for the Manchester virus where the capsid region of the polyprotein is fused to the RNA polymerase. Parkville virus however, demonstrates considerable sequence divergence from both the Manchester and Sapporo caliciviruses, providing the first indications that genetic diversity exists within caliciviruses of this previously homogeneous clade. On the basis of recent advances in the genetic characterization of members of the family *Caliciviridae,* we propose a new interim phylogenetic classification system in which Parkville virus would be included with Manchester and Sapporo virus as a separate group distinct from the small round-structured viruses (Norwalk-like viruses) that also cause diarrhea in humans. ***J. Med. Virol. 52:173–178, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

For the past two decades, electron microscopists studying fecal specimens from patients with gastroenteritis have used an "interim" classification scheme to distinguish morphologically "small round structured viruses" (SRSVs) such as Norwalk virus from viruses with distinct cup-shaped indentations or calices on their surface and a "Star of David" in the center that were called caliciviruses [Caul and Appleton, 1982]. These viruses also had distinguishing epidemiologic features; caliciviruses were found primarily in children less than 4 years which suggested that immunity might develop early in life [Cubitt and McSwiggan, 1987b; Grohmann, 1985; Matson et al., 1989, 1990; Monroe et al., 1991; Sakuma et al., 1981; Suzuki et al., 1979], whereas SRSVs were identified from adults as well as children and in outbreak settings traced to contaminated food or water [Khan et al., 1994; Kilgore et al., 1996; Kobayashi et al., 1991; Numata et al., 1994]. Immunity to SRSVs based on volunteer studies appeared to be short-lived without significant cross-protection between the many antigenically-distinct strains [Madore et al., 1990; Treanor et al., 1988, 1993; Wyatt et al., 1974].

The recent genetic characterization of both SRSV and calicivirus strains and the application of new diagnostic tools to detect and characterize viruses from clinical specimens has thrown into question the real distinctions between these two virus groups [Cubitt et al., 1994; Lambden et al., 1994; Liu et al., 1995; Matson

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et al., 1995]. The genomic organization of SRSVs with three overlapping large open reading frames (ORFs) is similar to that of feline calicivirus and formed the basis for their classification in the family *Caliciviridae* despite lacking typical calicivirus morphology [Jiang et al., 1990, 1993; Lambden et al., 1993, 1995; Lew et al., 1994a,b,c]. The tremendous genetic diversity observed between individual SRSV strains might then account for the susceptibility of people of all ages to different viruses in outbreak settings [Lew et al., 1994a,b; Moe et al., 1994; Monroe et al., 1993; Wang et al., 1994]. By contrast, the Manchester strain of calicivirus has a distinct genomic organization similar to rabbit hemorrhagic disease virus (RHDV) in which ORFs 1 and 2 are contiguous and encode a fused non structural-capsid polyprotein. The comparison of partial sequence information from two human viruses with typical calicivirus morphology demonstrated a >90% nucleotide identity between strains [Liu et al., 1995]. This homogeneity might explain the apparent immunity of children to calicivirus infections with a genetically similar strain.

We recently investigated an outbreak of acute viral gastroenteritis in adults that was traced to contaminated food and had epidemiologic features consistent with an outbreak due to typical SRSVs. When the etiologic agent was fully characterized, it was more closely related to the morphologic calicivirus strains than to SRSV strains. The sequence of this virus was quite distinct from all previously characterized strains. We are left with the prospect that human enteric caliciviruses are more genetically diverse than previously appreciated and that these viruses may behave like SRSVs, infecting adults and causing outbreaks indistinguishable from those caused by the SRSVs.

Description of the Outbreak

In May 1994, 46 cases of gastrointestinal illness were reported among adult staff at an elementary school in Parkville, Maryland, following a Parent Teacher Association-sponsored luncheon. Of 86 attendees who completed a questionnaire, 46 (53%) became ill with symptoms including diarrhoea (72%), stomach cramps (80%), nausea (78%), and vomiting (33%). The mean incubation period for the illness was 58 hr (range 5–100 hr) and mean duration of symptoms was 26 hr (range 12–46 hr). Epidemiologic investigation determined that the outbreak was most likely caused by contamination of one or more food items by ill employees at the catering company which prepared the luncheon. Bacterial cultures of 19 stool specimens were negative for *Salmonella* and *Shigella*.

MATERIALS AND METHODS

Parkville Outbreak Examination

Stool specimens (n = 7) and acute- and convalescent-phase sera (n = 14 pairs) from patients involved in the outbreak were sent to the Centers for Disease Control and Prevention (CDC) for viral testing. Fecal specimens were examined by electron microscopy (EM) and immune electron microscopy (IEM) was performed on matched stool and sera specimens from four patients [Humphrey et al., 1990]. Serum specimens were tested for IgG antibody to recombinant capsid proteins from Norwalk virus (rNV) [Monroe et al., 1993] and Toronto virus (rTV) [Leite et al., 1996] using a direct enzyme immunoassay (EIA) [Noel et al., manuscript submitted].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Nucleotide Sequencing

All stool specimens were screened for SRSVs by RT-PCR and Southern hybridization at CDC, according to the methods of Ando et al. [1995] and for caliciviruses by RT-PCR using primer SR33 and a new primer SR80 (5′ ^{178}TGG GAT TCT ACA CAA AAC CC^{197} 3′) based on the sequence of the Sapporo/82 strain [Matson et al., 1995] under conditions of low stringency (42°C anneal). The 307-bp RT-PCR product generated from one specimen (CDC 94012584) provided sequence data to synthesize specific Parkville virus primers SR129 (5′ ^{293}TCA CCA TAA GTG TGA ACA GTC TC^{271} 3′) (negative sense, numbers refer to sequence of the Parkville virus genome, U73124) and SR84 (5′ ^{57}CAG CCC ACT AGT GTC ATG TG^{76} 3′) (positive sense) with an expected amplicon size of 237-bp.

Parkville Virus Genome Sequencing

An aliquot of the total stool viral RNA from specimen CDC 94012584 was sent to the UK for nucleotide sequencing. Primers designed on two amino acid motifs (YSKWDST and GLPSGMP) which are conserved in RHDV, FCV, and the Manchester strain of calicivirus were used to amplify a section of the RNA polymerase region of the genome. The eight-fold degenerate primer CV1 (5′ TAC TCC AAR TGG GAY TCS AC 3′) and the four-fold degenerate primer CV2 (5′ GGC ATC CCA GAT GGY AGR CC 3′) were used to generate a 183-bp amplicon which provided sequence data to synthesize the specific Parkville virus primer WV5(5′ ^{106}GGC TAC CTC AAT GAC ATT AA^{125} 3′). The 3′ terminal half of the Parkville virus genome was subsequently amplified as a single 3 kb amplicon using oligo dT_{25} and the specific primer WV5, preliminary sequence data was used to synthesize specific primers for direct sequencing of both strands of amplified cDNA as previously described [Liu et al., 1995].

Sequence Analysis

Computer analyses of sequence data were performed using the Lasergene software (DNASTAR Inc., Madison, WI), the GCG suite of programs [Genetics Computer Group, 1994], and the Phylogenetic Analysis Using Parsimony (PAUP) program, version 3.0s [Swofford, 1991]. The EMBL/Genbank accession number for Parkville virus is U73124.

RESULTS

Parkville Outbreak Examination

Viruses 28-30nm in size were detected by EM in five of seven stool specimens. Close examination of electron

micrographs revealed the presence of calicivirus-like surface depressions on some of the viral particles. Immune electron microscopy (IEM) showed aggregation of virus with the homologous convalescent-phase serum, but not by the acute-phase serum, indicating that virus infection was associated with disease. No seroconversions to either rNV or rTV capsid antigens were measured in the serum pairs from any of the 14 patients.

RT-PCR and Nucleotide Sequencing

Consistent with the lack of seroconversions to either of the expressed capsid antigens, all stool specimens were repeatedly negative when tested using our standard methods of RT-PCR and Southern hybridization specific for SRSVs, suggesting that the infecting virus differed significantly from common SRSV strains. Sequence data for the 307-bp amplicon generated using our HuCV-Sapporo RT-PCR showed that it contained the RNA-dependent RNA polymerase motifs GLPSG and YGDD characteristic of members of the family *Caliciviridae.* Subsequent phylogenetic analysis confirmed that this virus, designated as Parkville virus, was a calicivirus strain related to, but distinct from, the Sapporo/82 and Manchester viruses. RT-PCR using the Parkville virus specific primers (SR129 and SR84) was used to successfully amplify a 237-bp amplicon from all seven stool specimens from the outbreak.

Parkville Virus Genome Sequencing

The sequence data obtained for the 3′terminal portion of the Parkville virus viral genome consisted of 3085 nucleotides excluding the polyadenylate tail and covered a region from within the RNA polymerase extending to the extreme 3′ terminus of the viral genome (Genbank Accession number U73124). Nucleotide sequences across the RNA polymerase region of Parkville virus obtained in the US and the UK were 100% concordant. Analysis of predicted open reading frames indicated a genome organisation identical to that reported for Manchester virus where the capsid region of the polyprotein is fused to the RNA polymerase [Liu et al., 1995].

The predicted translation products of Parkville virus were compared with those of Manchester virus, the partial sequence available for Sapporo virus and sequences of several reference animal calicivirus and SRSV strains. The RNA polymerase partial amino acid sequence, encoded at the amino end of the ORF 1 polyprotein, showed 82% sequence identity with that of Manchester virus over 264 residues (Fig. 1A). However the sequence identity was significantly lower when compared to FCV (43%), RHDV (35%), and NV (31%). In the 120 amino acid region of overlap with the partial sequence of the Sapporo virus (residues 1–120 of Fig. 1A), there are 23 differences between the Manchester and Parkville viruses (81.1% identity) vs. only one difference between the Manchester and Sapporo viruses (99.2% identity). A phylogenetic analysis of this 140 amino acid region comparing reference strains of *Caliciviridae* confirms that Parkville virus is distinct from both the Sapporo and Manchester viruses (Fig. 2). This figure also demonstrates the four distinct genetic lineages of caliciviruses, as previously reported [Matson et al., 1995]. In our analysis, the calicivirus strains were more closely related to RHDV than to FCV, although the bootstrap value for this distinction was only 56%.

The Parkville virus capsid, encoded at the carboxy end of the ORF 1 polyprotein, was predicted to be 10 amino acids longer (571 aa) than that of Manchester virus (561 aa) with an overall sequence identity of 80% (Fig. 1A). The C-terminal half of the viral capsid gene showed a greater sequence divergence (73% identity) than the N-terminal region (85% identity).

The 3′ terminal ORF identified in all members of the *Caliciviridae* analysed to date was also present in Parkville virus (Fig. 1B). The predicted translation product from this ORF was 163 amino acids and showed 72% amino acid identity with Manchester virus (165 aa).

A unique feature of the Manchester virus is a small ORF which overlaps the N-terminal region of the capsid protein gene [Liu et al., 1995]. The biologic role for this ORF remains in question, since no homolog has been detected in any of the animal caliciviruses or SRSVs. The Parkville virus sequence reported here contains a similar small ORF, which is predicted to encode a polypeptide of 163 amino acids, two longer than that of the Manchester strain (Fig. 1C). Strikingly, the predicted translation products from this ORF showed the greatest sequence divergence, with only 61% identity between the two viruses. Analysis of the nucleotide sequence in this region of potential gene overlap revealed that compared to Manchester virus, 42 of the capsid codons are altered at the third base position and that only three of these nucleotide changes result in an amino acid substitution. The effect of these nucleotide changes on the +1 reading frame is to alter the second base of the codons in the small ORF, resulting in a greater number of amino acid substitutions between the two viruses. That the silent third base changes in the capsid gene are not selected against in the region of overlap suggests that the small ORF may not encode a functional protein.

DISCUSSION

The etiologic agent of this epidemic was not a typical SRSV commonly associated with outbreaks of acute gastroenteritis in adults but a virus with the morphology and genomic organization of a calicivirus commonly found in children with diarrhea. Characterization of the Parkville virus showed it to be a novel strain with both epidemiologic and genetic properties that distinguished it from previously identified caliciviruses (e.g., Manchester virus and Sapporo virus). However, unlike these caliciviruses which are genetically similar, the Parkville virus was a genetically divergent strain with a diversity similar to that seen for the two separate genogroups of SRSVs. This suggests that caliciviruses of the Sapporo group may also have separate

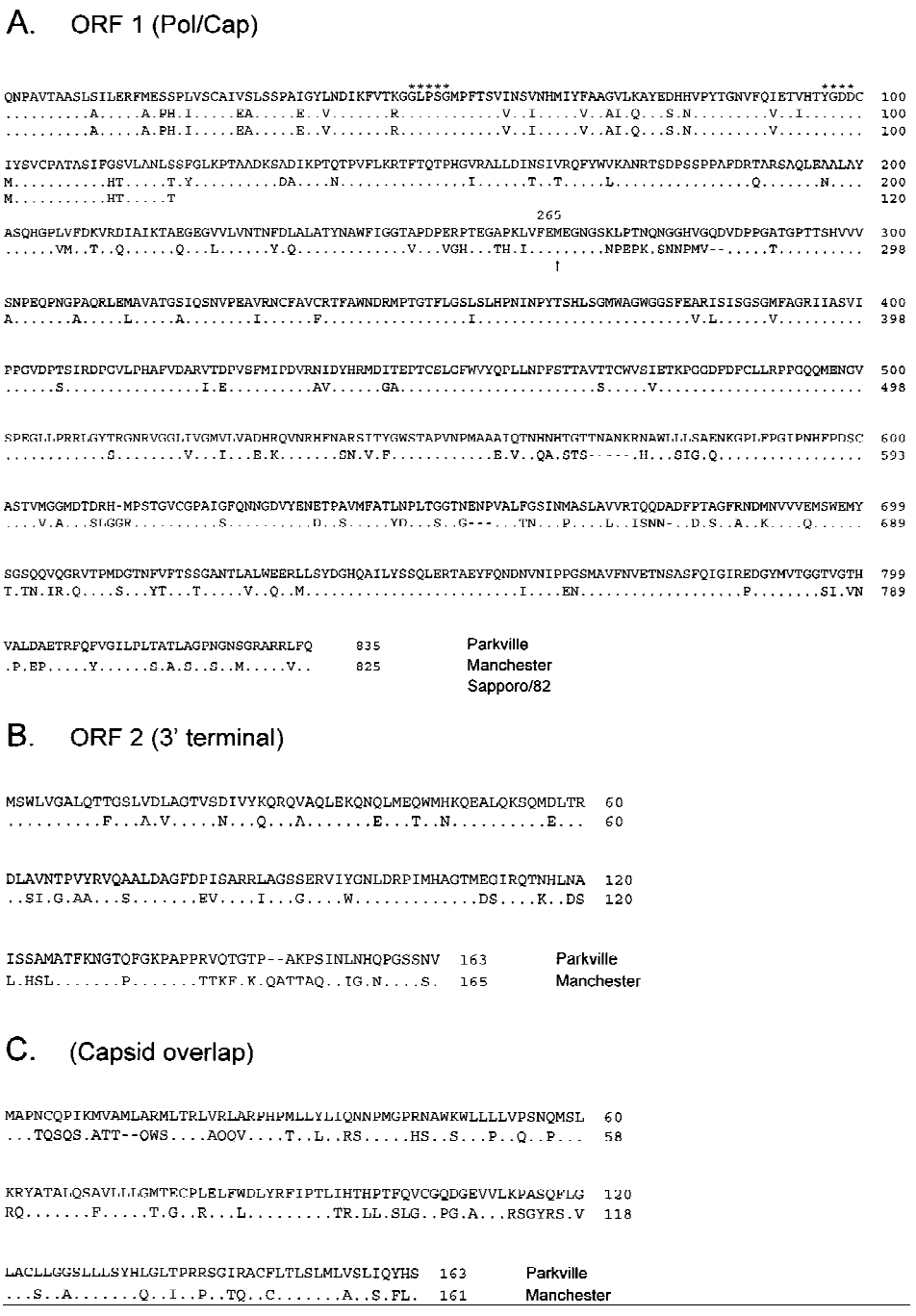

Fig. 1. Comparison of the predicted amino acid sequences of the Parkville, Manchester, and Sapporo (where available) caliciviruses. Positions of identity between the viruses are indicated with a dot. Accession numbers for the sequences used for the comparison are: Manchester virus (X86560) and Sapporo/82 (S77903). **A**: A comparison of the partial sequence of the ORF 1 polyproteins. Conserved amino acid motifs in the RNA-dependent RNA polymerase are indicated by asterisks. The upwards facing arrow indicates the putative N-terminus of the capsid protein at residue 265. **B**: A comparison of ORF2. **C**: A comparison of the small open reading frame which overlaps the amino terminus of the capsid gene. Hyphens indicate the two amino acid deletions in Manchester virus.

genogroups and points to the need to further examine other strains of morphologically similar caliciviruses to understand the full genetic diversity of this group.

Identification of small round fecal viruses by electron microscopy has traditionally been based upon an "interim" scheme of classification [Caul and Appleton, 1982] which distinguished caliciviruses, having a "Star of David" surface morphology, from small round structured viruses, with an amorphous surface and ragged outline. Due mainly to a lack of widely available and standardized immune reagents, several investigators developed independent and often conflicting schemes for further distinguishing viruses within these groups by using immune reagents derived from infected pa-

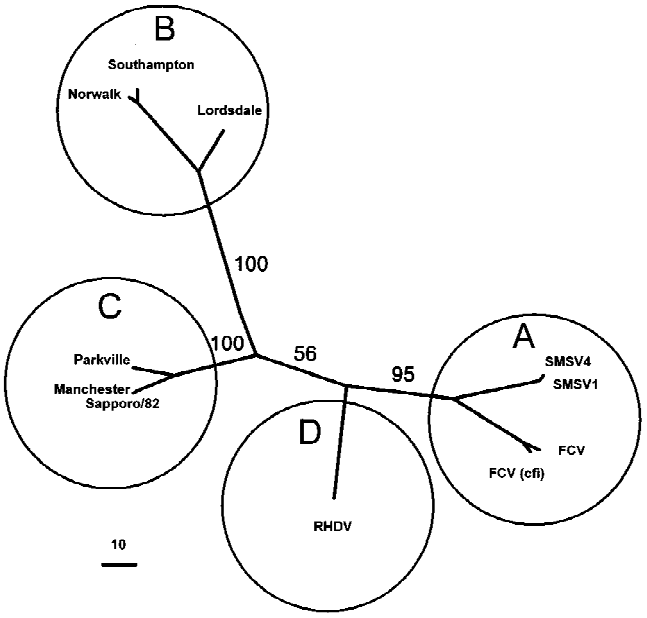

Fig. 2. Phylogenetic analysis of the RNA polymerase of representative members of *Caliciviridae.* A 120 amino acid region including the GLPSG and YGDD amino acid motifs was analyzed by using the PAUP program [Swofford, 1991]. Accession numbers for the sequences used for the comparison are: Norwalk (M87661), Southampton (L07418), and Lordsdale (X86557) SRSV strains, Manchester (X86560), Sapporo/82 (S77903), and Parkville (U73124) calicivirus strains, FCV (M86379, U13992), SMSV1 (U15301), SMSV4 (U15302), and RHDV (M67473). The scale bar indicates 10 amino acid changes. Bootstrap values are indicated for main branches.

tients [Cubitt et al., 1987a; Lewis, 1990; Lewis et al., 1995; Okada et al., 1990]. The resulting nomenclature confusion was compounded by the analysis of nucleotide sequence information for several SRSV strains that indicated that these viruses should be classified within the family *Caliciviridae* [Jiang et al., 1990, 1993; Lambden et al., 1993, 1995; Lew et al., 1994a,b]. Subsequently, the term human calicivirus has been variously used to refer either strictly to viruses with the typical or classic surface morphology or, more broadly, to all human viruses belonging to the family *Caliciviridae.* Additionally, although the well characterized Sapporo strain of calicivirus was shown to be genetically distinct from all characterized animal caliciviruses and SRSV strains, some viruses with the Star of David surface morphology have been shown to cluster genetically with SRSV strains [Cubitt et al., 1994; Stene-Johansen et al., 1996]. These results raise questions about the classification of small round fecal viruses solely on the basis of surface morphology observed in the electron microscope.

The current official classification within the family *Caliciviridae* includes a single genus, calicivirus, with vesicular exanthema of swine virus (VESV) as the prototype strain [Cubitt et al., 1995]. As analysis of nucleotide sequence information from several representative caliciviruses has become available, it is increasingly clear that there are significant differences in the genome organization and predicted phylogenetic relationships between members of this family. One group, represented by feline calicivirus (FCV) and partial sequence suggests it includes VESV, encodes three overlapping reading frames, with the nonstructural and capsid proteins in separate frames. A second group, represented by Norwalk virus, has a similar genetic organization, but is a distinct genetic lineage, or clade, by phylogenetic analysis [Jiang et al., 1990, 1993]. A third group, represented by the Manchester and Sapporo caliciviruses encodes only two large open reading frames, with the nonstructural and capsid proteins encoded in phase [Liu et al., 1995]. A fourth group, represented by rabbit hemorrhagic disease virus (RHDV), has a genome organization similar to that of the Manchester and Sapporo viruses but is a distinct phylogenetic clade. The fifth group, represented by hepatitis E virus (HEV), is currently included within *Caliciviridae,* but this is uncertain because of the similarity the nonstructural gene organization shows to that of rubiviruses. Although these groups may eventually be classified as separate genera, we propose a new interim phylogenetic classification as groups A–D, respectively. Thus, FCV would be referred to as a group A calicivirus, Norwalk virus as a group B calicivirus, Manchester and Parkville viruses as group C caliciviruses and RHDV as a group D calicivirus. While a classification system based upon neutralization or antigenic reactivity may be preferable to one based on genetic typing, the inability to grow many of these human viruses in cell culture and the lack of standardized reagents for comparing antigenicity preclude either of these typing systems.

These observations raise several questions for future study of human calicivirus disease. Are the differences in the epidemiology of the Parkville and Sapporo calicivirus strains related to their genetic differences? Are other Parkville-like group C caliciviruses commonly associated with outbreaks of gastroenteritis in adults? These results challenge us to develop better molecular detection methods for caliciviruses and to apply them to the search for caliciviruses as a cause of epidemics of gastroenteritis in older children and adults, particularly in those outbreaks from which a SRSV can not be identified.

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